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DOCKET NO. : UPN-3904

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Cameron J. Koch; Sydney M. Evans; Chyng-Yann Shiue; Ian R. Baird; Kirsten A. Skov; William R. Dolbier, Jr.; An-Rong Li and Brian R. James

Serial No.: Not Assigned Yet

Group Art Unit: Not Assigned Yet

Filing Date: Herewith

Examiner: Not Assigned Yet

For: Detection of Hypoxia

EXPRESS MAIL LABEL NO: EL531436721US
DATE OF DEPOSIT: August 25, 2000

Box ☒ Patent Application

☐ Provisional ☐ Design

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☒ A Utility Patent Application under 37 C.F.R. 1.53(b).

It is a continuing application, as follows:

☐ continuation ☒ divisional ☐ continuation-in-part of prior application number 09/123,300.

☐ A Provisional Patent Application under 37 C.F.R. 1.53(c).

☐ A Design Patent Application (submitted in duplicate).

Including the following:

☐ Provisional Application Cover Sheet.

☐ New or Revised Specification, including pages ___ to ___ containing:

- ☐ Specification
- ☐ Claims
- ☐ Abstract
- ☐ Substitute Specification, including Claims and Abstract.

☐ The present application is a continuation application of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Since none of those amendments incorporate new matter into the parent application, the present revised Specification also does not include new matter.

☐ The present application is a continuation application of Application No. _____ filed _____, which in turn is a continuation-in-part of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Although the amendments in the parent C-I-P application may have incorporated new matter, since those are the only revisions included in the present application, the present application includes no new matter in relation to the parent application.

☒ A copy of earlier application Serial No. 09/123,300 Filed July 28, 1998, including Specification, Claims and Abstract (pages 1 - 35), to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

☒ Please enter the following amendment to the Specification under the Cross-Reference to Related Applications section (or create such a section) : "This Application:

☐ is a continuation of ☒ is a divisional of ☐ claims benefit of U.S. provisional Application Serial No. 09/123,300 filed July 28, 1998

Signed Statement attached deleting inventor(s) named in the prior application.

Signed Statement attached deleting inventor(s) named in the prior application.

☒ A Preliminary Amendment.

☒ Five Sheets of ☐ Formal ☒ Informal Drawings.

☐ Petition to Accept Photographic Drawings.

☐ Petition Fee

☒ An ☒ Executed ☐ Unexecuted Declaration or Oath and Power of Attorney.

☐ An Associate Power of Attorney.

☐ An ☐ Executed ☐ Copy of Executed Assignment of the Invention to The Trustees of the University if Pennsylvania

☐ A Recordation Form Cover Sheet.

☐ Recordation Fee - $40.00.

☒ The prior application is assigned of record to the Trustees of the University of Pennsylvania.

☐ Priority is claimed under 35 U.S.C. § 119 of Patent Application No. _____ filed _____ in _____ (country).

☐ A Certified Copy of each of the above applications for which priority is claimed:

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____ .

☒ An ☐ Executed or ☒ Copy of Executed Earlier Statement Claiming Small Entity Status under 37 C.F.R. 1.9 and 1.27

☒ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____ , said status is still proper and desired in present case.

☐ Diskette Containing DNA/Amino Acid Sequence Information.

☐ Statement to Support Submission of DNA/Amino Acid Sequence Information.

☐ The computer readable form in this application _____, is identical with that filed in Application Serial Number _____, filed _____. In accordance with 37 CFR 1.821(e), please use the ☐ first-filed, ☐ last-filed or ☐ only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is ☐ included in the originally-filed specification of the instant application, ☐ included in a separately filed preliminary amendment for incorporation into the specification.

☐ Information Disclosure Statement.

- ☐ Attached Form 1449.
- ☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

☐ A copy of Petition for Extension of Time as filed in the prior case.

☐ Appended Material as follows: _____.

☒ Return Receipt Postcard (should be specifically itemized).

☐ Other as follows: _____

_____

_____.

FEE CALCULATION:

☒ Cancel in this application original claims 1-19 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

			SMALL ENTITY		NOT SMALL ENTITY	
			RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION			$75.00	$	$150.00	$
DESIGN APPLICATION			$155.00	$	$310.00	$
UTILITY APPLICATIONS BASE FEE			$345.00	$345	$690.00	$
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS						
	No. Filed	No. Extra				
TOTAL CLAIMS	13- 20 =	0	$9 each	$	$18 each	$
INDEP. CLAIMS	2- 3 =	0	$39 each	$	$78 each	$
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			$130	$	$260	$
ADDITIONAL FILING FEE				$0		$
TOTAL FILING FEE DUE				$345.00		$

A Check is enclosed in the amount of $345.00.

☒ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.

☐ The foregoing amount due.

☒ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.

☒ Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).

☐ The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-identified application during the pendency of this application. The Commissioner is

further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: August 25, 2000

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Registration No. 44,121

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Applicant or Patentee: Alexander V. Kachur, Sydney M. Evans, Chyng-Yann Shiue, Ian R. Baird, Kirsten A. Skov, William R. Dolbier, Jr., An-Rong Ki, and Cameron J. Koch

Serial or Patent No.: Not Yet Assigned

Attorney's Docket No.: UPN-3388

Date Filed or Issued: Herewith

For: Detection of Hypoxia

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Trustees of the University of Pennsylvania

ADDRESS OF ORGANIZATION: 3700 Market Street
Philadelphia, PA 19104

TYPE OF ORGANIZATION:

(XX) UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

( ) TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))

( ) NONPROFIT SCIENTIFIC OR EDUCATIONAL ORGANIZATION QUALIFIED UNDER A NONPROFIT ORGANIZATION STATUTE OF A STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

( ) WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA

( ) WOULD QUALIFY AS A NONPROFIT SCIENTIFIC OR EDUCATIONAL ORGANIZATION QUALIFIED UNDER A NONPROFIT ORGANIZATION STATUTE OF A STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled Detection of Hypoxia by inventor(s) Alexander V. Kachur, Sydney M. Evans, Chyng-Yann Shiue, Ian R. Baird, Kirsten A. Skov, William R. Dolbier, Jr., An-Rong Ki, and Cameron J. Koch described in

(XX) the specification filed herewith.

( ) application serial no. _____, filed _____.
( ) patent no. _____, issued _____.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME:
ADDRESS:
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING	Evelyn McConathy, Esquire
TITLE IN ORGANIZATION	Director, Intellectual Properties
ADDRESS OF PERSON SIGNING	3700 Market Street, Suite 300 Philadelphia, PA 19104

Evelyn H. McConathy
SIGNATURE

July 28, 1998
DATE

Docket No: UPN-3904 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Koch et al.

Serial No: Not Assigned Yet **Group Art Unit: Not Assigned Yet**

Filed: Herewith **Examiner: Not Assigned Yet**

For: Detection of Hypoxia

EXPRESS MAIL LABEL NO: EL531436721US
DATE OF DEPOSIT: August 25, 2000

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

In the specification:

Under "RELATED APPLICATIONS" after "filed on February 8, 1996" please add -- now U.S. Pat. No. 5,843,404, issued December 1, 1998, which is a divisional of application Serial No.286,065 filed August 4, 1994, now U.S. Pat. No. 5,540,908, issued July 30, 1996, which is a continuation in part of application Serial No. 978,918, filed November 19, 1992, now abandoned.--

In the claims:

Please add the following claims:

27. (New) The method of claim 20 wherein the halogen is fluorine.

28. (New) The method of claim 20 wherein R_2 is $CH_2CF_2CH_2F$.

29. (New) The method of claim 20 wherein R_2 is $CH_2CF_2CHF_2$.

30. (New) The method of claim 20 wherein R_2 is CH_2CHFCH_2F.

31. (New) The method of claim 20 wherein R_2 $CH_2CHFCHF_2$.

32. (New) The method of claim 20 wherein R_2 is $CH_2CH_2CHF_2$.

In the abstract:

Please delete the word "Novel" in the first line of the abstract.

REMARKS

Upon entry of the proposed amendment claims 20-32 will be pending. Support for the new claims can be found in the specification at, for example, page 10, line 11 through page 11, line 4. No new matter has been added.

Applicants believe that the claims are in condition for allowance. An early Office Action to that effect is, therefore, earnestly solicited.

Respectfully submitted,

Maureen S. Gibbons
Registration No. 44,121

Date: 8/25/00

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DETECTION OF HYPOXIA

RELATED APPLICATIONS

This application is a continuation in part of application Serial No. 08/598,752, filed on February 8, 1996.

FIELD OF THE INVENTION

This invention generally relates to a class of nitroaromatic compounds that, when activated by reductive metabolism, bind to hypoxic cells. This reductive metabolism and binding increase as the oxygen concentration of cells decreases, which enables these compounds to be used as indicators of hypoxia. The present invention presents novel nitroaromatic compounds; immunogenic conjugates comprising the novel nitroaromatic compounds and proteins; and monoclonal antibodies specific for the novel nitroaromatic compounds of the invention, their protein conjugates, their reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention. The invention is further directed to methods for detecting levels of low oxygen in tissue. Detection may be done directly using methods such as imaging techniques involving specific isotopes attached to the nitroaromatic drug, or indirectly using the monoclonal antibodies (mAbs) in immunohistological assays. Still further, the present invention is directed to kits for performing the methods of the invention.

BACKGROUND OF THE INVENTION

One of the most important goals in oncology is the identification and elimination of treatment resistant cells; hypoxic cells are the most familiar examples of this type of cell. Kennedy, *et al.*, *Biochem. Pharm.* **1980**, *29*, 1; Moulder, *et al.*,*Int. J. Radioat. Oncol. Biol. Phys.* **1984**, *10*, 695; Adams, *Cancer*, **1981**, *48*, 696. Hypoxic cells are seldom found in normal tissues, and are generally found only in conjunction with certain tumors, vascular diseases, or after a stroke.

As certain tumors enlarge, the tissue often outgrows its oxygen and nutrient supply because of an inadequate network of functioning blood vessels and capillaries. Although the cells deprived of oxygen and nutrients may ultimately die, at any given time a tumor may produce viable hypoxic cells. These hypoxic cells, although alive, have very low oxygen concentrations because of their remoteness from the blood vessels.

The level of molecular oxygen has important implications in disease diagnosis and prognosis. In medical oncology, for example, hypoxic cells in solid tumors may be highly resistant to killing by some forms of chemotherapy. When chemotherapeutic agents are administered to patients, the agents are carried through the functioning blood vessels and capillaries to the target tissue. Because hypoxic tissue lacks a fully functioning blood supply network, the chemotherapeutic drugs may never reach the hypoxic cells; instead, intervening cells scavenge the drug. The result is that the hypoxic cells survive and recurrence of the tumor is possible. Kennedy, *et al.*, *supra*.

Tissue hypoxia also hinders the effectiveness of radiation therapy, especially of neoplasms. Radiation treatment is most effective in destroying oxygen containing cells because oxygen is an excellent radiation sensitizer. The presence of hypoxic cells impedes this treatment because their low oxygen concentration renders the ionizing radiation relatively ineffective in killing the cancerous cells. Therefore, hypoxic cells are more likely to survive radiation therapy and eventually lead to the reappearance of the tumor. The importance of hypoxic cells in limiting radiation responsiveness in animal tumors is well known, Adams, supra; Moulder, *et al.*, *supra*; Chapman, *et al.*,"*The Fraction of Hypoxic Clonogenic Cells in Tumor Populations*," *in Biological Bases and Clinical Implications of Tumor Radioresistance*

61, G.H. Fletcher, C. Nevil, & H.R. Withers, eds., **1983**. Studies have revealed that such resistant cells greatly affect the ability of radiation and chemotherapy to successfully sterilize tumors in animals. Substantial work since that time has shown similar problems in human tumors. Despite the progress in animal studies regarding the identification of hypoxic cells, limited success has been achieved in humans. One reason for this disparity may relate to differences in tumor growth and other host related factors, but in addition, there has been no suitably accurate method to assess tissue oxygen at a sufficiently fine resolution.

Venous oxygen pressure is generally ~35 Torr, an oxygen level providing nearly full radiation sensitivity. As the oxygen level decreases below 35 Torr, radiation resistance gradually increases, with half-maximal resistance at about 3.5 Torr, and full resistance at about 0.35 Torr. Therefore, it is necessary to measure much lower oxygen levels than are usually encountered in normal tissue. Current technology does not meet this need. Oxygen partial pressure measured using current techniques often yields an average value for large numbers of neighboring cells. This is a severe impediment for detection and diagnosis because histological evaluation of solid tumors suggest that important changes in cellular oxygen can occur over dimensions of even a few cell diameters. Urtasun, *et al.*, *Br. J. Cancer*, **1986**, *54*, 453. Nitroheterocyclic drugs have been under extensive investigation as oxygen indicators. It is known that this class of compounds has the potential for resolution at the cellular level and can provide sufficient sensitivity to monitor the low oxygen partial pressures described above. This technique involves the administration of nitroaromatic drugs to the tissue of interest. The drugs undergo bioreductive metabolism at a rate which increases substantially as the tissue's oxygen partial pressure decreases. The result of this bioreductive metabolism is that reactive drug products are formed which combine chemically to form adducts with predominantly cellular proteins. Because the metabolic binding of these compounds to cellular macromolecules is inhibited by oxygen, these compounds bind to hypoxic cells in preference to normal, healthy, oxygen-rich tissue. This preferential metabolic binding, or adduct formation, provides a measure of the degree of hypoxia. Koch, *et al.*, *Int. J. Radiation Oncology Biol. Phys.*, **1984**,*10*, 1327.

Misonidazole (MISO) 3-methoxy-1-(2-nitroimidazol-1-yl)-2-propanol, and certain of its derivatives have been under extensive investigation as indicators of hypoxia in mammalian tissue. Chapman, *et al.*, *Int. J. Radiat. Oncol. Biol. Phys.*, **1989**,*16*, 911; Taylor, *et al.*, *Cancer Res.*, **1978**, *38*, 2745; Varghese, *et al.*,*Cancer Res.*, **1980**, *40*, 2165. The ability of certain misonidazole derivatives to form adducts with cellular macromolecules, referred to as binding throughout this application, has formed the basis of various detection methods.

For example, ^{3}H or ^{14}C labeled misonidazole has been used *in vitro* and *in vivo*, with binding analyzed by liquid scintillation counting or autoradiography. Chapman, **1984** *supra*; Urtasun, **1986,** *supra*; Franko, *et al.*, *Cancer Res.*, **1987**, 47, 5367. A monofluorinated derivative of misonidazole has utilized the positron emitting isotope F18 for imaging bound drug *in vivo*, Rasey, *et al.*, *Radiat. Res.*, **1987**, *111*,292. The method of the preparation of the PET derivative of ethanidazole was described in Tewson T.J. Synthesis of [^{18}F] Fluoroetanidazole: a potential new tracer for imaging hypoxia. *Nuclear Medicine & Biology*, 24(8):755-60, 1997.

A hexafluorinated derivative of misonidazole (1-(2-hydroxy-3-hexafluoro-isopropoxy-propyl)-2-nitroimidazole has been assayed directly (no radioactive isotopes) via nuclear magnetic resonance spectroscopy (NMR or MRI) techniques. Raleigh, *et al.*, *Int. J. Radiat. Oncol. Biol. Phys.*, **1984**, *10*, 1337. Polyclonal antibodies to this same derivative have allowed immunohistochemical identification of drug adducts. Raleigh, *et al.*, *Br. J. Cancer*, **1987**, *56*, 395. An iodine isotope has been incorporated into another azomycin derivative, azomycin arabinoside, allowing radiology techniques of detection. Parliament, *et al.*, *Br. J. Cancer*, **1992**, *65*, 90.

A fluorescence immunohistochemical assay for detecting hypoxia is described in the literature. Raleigh, *et al.*, **1987**, *supra*. A method for preparing immunogenic conjugates for use in such assays is broadly disclosed in U.S. Patent No. 5,086,068, issued to Raleigh, *et al.*, on February 4, 1992 ("Raleigh patent"). The Raleigh patent describes a method for preparing an immunogenic conjugate comprising a known fluorinated misonidazole derivative and an immunogenic carrier protein, hemocyanin. The compound used in this method (CCI-103F) was a hexafluorinated derivative of 2-nitroimidazole

misonidazole, described above in connection with NMR studies.

The resulting conjugate is used to raise rabbit polyclonal antibodies specific for the misonidazole derivative. Fluorescence immunohistochemical studies showed that the polyclonal antibodies bound to hypoxic (central) regions of spheroids (a multicellular aggregate of cells in tissue culture having some properties more closely related to tumors) and tumor sections in patterns similar to those revealed by audioradiographic studies using radioactive drug alone, i.e. without polyclonal antibodies.

However, polyclonal antibodies are plagued by numerous difficulties such as cross-reactivity, lack of specificity, insensitivity, inability to purify the actual antibodies of interest, and highly unstable supply.

The Raleigh patent's technology, of conjugating a small antigen to a large carrier protein to elicit an immune response, is a central basis of antibody production and is well known in the art. Those skilled in the art would also appreciate that nitroaromatics must be activated by chemical or biochemical reduction to cause adducts to form with cellular macromolecules. Further, it has not been possible to produce monoclonal antibodies using the methods described in the Raleigh patent and paper (Raleigh *et al.*,**1987**, *supra*).

The Raleigh patent discloses immunogenic conjugates useful for producing polyclonal antibodies, but data generated using the patent's teachings has produced variable results, problematic in a detection technique. Furthermore, independent experimentation performed according to the Raleigh patent's methods did not reproduce the high degree of conjugation between the misonidazole derivatives and the protein as was claimed. *See, e.g.*, U.S. Patent No. 5,540,908, the disclosures of which are herein incorporated by reference in their entirety.

The bioreductive drug assays described above do not directly measure oxygen partial pressure, even though this is the required value, using the example of radiation therapy to predict radiation response. Rather, the assays measure adduct formation, a biochemical process which is inhibited by oxygen. The data generated using these methods has shown that the degree of inhibition by oxygen varies substantially from tissue to tissue. Franko, *et al.*, **1987**, *supra*. Furthermore, the maximum rate of adduct formation in the complete absence of oxygen is also highly variable from tissue to tissue, as is the maximum percentage of

inhibition by oxygen, Koch, *in Selective Activation of Drugs by Redox Processes*, Plenum Press, pp. 237-247, Adams, *et al.*, eds, New York, **1990**. Another way of expressing these limitations is that the bioreductive formation of nitroaromatics provide only a relative indication of varying oxygen levels, but is inadequate at providing an absolute measurement of oxygen partial pressure because there are several factors which affect adduct formation in addition to changes in oxygen, non-oxygen-dependent factors. Additionally, the choice of nitroaromatic drug affects the variability related to the non-oxygen-dependent factors.

Early research efforts (*i.e.*, before the invention claimed in U.S. Patent No. 5,540,908 on Nov. 19, 1992) had focused on misonidazole and certain of its derivatives. However, misonidazole is the most susceptible of several drugs tested to non-oxygen-dependent variations in adduct formation. Koch, *Selective Activation*, *supra*. Other problems relate to various physicochemical properties of existing drugs, all of which can influence the non-oxygen dependent variations in adduct formation. For example, the hexafluorinated misonidazole derivative described above had a high degree of insolubility.

Although 2-nitroimidazoles labeled with radiochemical tracers such as tritium and ^{14}C provide a sensitive method for detecting tissue hypoxia using autoradiographic methods, the biohazards and costs associated with these techniques are a significant drawback. The amount of radioactivity associated with the administration of such labeled drugs, which still requires a tissue biopsy, becomes a substantial problem in animal studies and an even greater problem in humans where 30 millicuries of tritiated drug are typically used. Urtasun, *et al.*, **1986**, *supra*. ^{14}C is prohibitively expensive and causes unacceptable radiation exposures. The use of such radioactive tracers is generally not acceptable because of the stringent requirements associated with handling radioactive tissues and bodily fluids. There are also practical limitations to the use of radioactive tracers. For example, the delay required for audioradiographic analysis of the tissue sections, often several weeks, is a very serious impediment to the rapid analysis required in treatment determination. Moreover, toxicity problems associated with certain misonidazole derivatives resulted in the drug being administered at a relatively low concentration, which decreased detection sensitivity. Thus, to utilize the high sensitivity of radioactive drug methods, short-lived isotopes analyzable by non-invasive methods such as PET and SPECT are preferred; there is still a need for such

methods.

Many human and animal diseases are characterized by the pathological formation of tissue hypoxia and ischemia. Hypoxic cells in solid tumors have been associated with treatment resistance by radiation, Moulder, *supra*, and some forms of chemotherapy, Kennedy, *supra*. Treatment of such conditions can only be optimized by determining the extent and degree of hypoxia in the affected tissues of individual patients. Accordingly, there is a great oncological need to identify hypoxic cells.

While biopsy-based methods are applicable to many forms of analysis in tumors, non-invasive assays are required for diseases of normal tissue such as heart attack and stroke. Again, one must employ techniques such as MRS/MRI, PET, and SPECT.

Previous studies have exemplified the determination of hypoxia in normal and diseased tissues by detecting metabolites of drugs named 2 (2-nitro-1H-imidazol-1-yl) -N-(2,2,3,3,3-pentafluoropropyl) acetamide (hereinafter referred to as EF5) and 2(2-nitro-1H-imidazol-1-yl)-N-(3,3,3-trifluoropropyl) acetamide (hereinafter referred to as EF3). *See* U.S. Patent Nos. 5,540,908, issued to Koch *et al*, the disclosures of which are herein incorporated by reference.

Notwithstanding the significant advances already attained with EF5 and EF3, there still remains a need in the art for compounds that are useful in noninvasive imaging techniques, such as MRI and PET. *See also Detection of Hypoxic Cells by Monoclonal Antibody Recognizing 2-Nitroimidazole Adducts*, *Cancer Res.*, **1993**, *53*, 5721-76, the disclosures of which are herein incorporated by reference. It is highly desirable to be able to assay for the presence of hypoxic cells in an animal or human tumor, and to do so predictably and without the concomitant hazards associated with radioactivity. The compounds and methods of the claimed invention address these, as well as other, needs in the art.

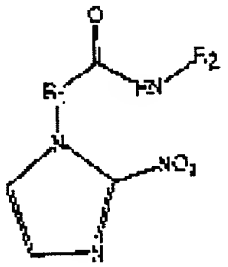
SUMMARY OF THE INVENTION

This invention presents novel nitroaromatic compounds; immunogenic conjugates comprising the novel nitroaromatic compounds and proteins; and monoclonal antibodies specific for the novel nitroaromatic compounds of the invention, their protein conjugates, their reductive byproducts, and adducts formed between mammalian hypoxic cells

and the compounds of the invention. The novel compounds' protein conjugates, reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention may be generally referred to as compositions throughout this application. The novel compounds and compositions of the invention, and the methods according to this invention, provide the basis for sensitive and precise methods for detecting tissue hypoxia.

The present invention presents a novel class of compounds, similar in core structure to etanidazole but having new side chains that make them much more predictable oxygen indicators and much more amenable to immunohistochemical and other noninvasive assays. The novel compounds and compositions of the invention and the corresponding methodologies provide techniques for measuring the degree of hypoxia in mammalian tumors with a precision and sensitivity that has not been achieved before. These novel compounds and compositions may be used to detect hypoxia using standard nuclear medical procedures with a consistency not previously observed in the art. These novel compounds also provide the basis for immunological assays. These novel compounds thus afford the opportunity to study and compare their biodistribution using both microscopic (immunohistochemical) and macroscopic (immunological, MRS/MRI, PET) methods at drug concentrations appropriate for each method, but also to compare methods at constant drug concentration. This allows for much new information on the pharmacology and biodistribution of such molecules. It is seldom appreciated that drug pharmacology at drug concentrations used in typical nuclear medicine procedures, picomolar to micromolar range, may have little in common with drug pharmacology at much higher concentrations.

The novel class of compounds of this invention have the general structure depicted below



wherein R_1 is CH_2; and R_2 has the formula $CH_2CX_2CHX_2$, wherein X is halogen or hydrogen and at least 1 carbon atom of said R_2 group is substituted with at least one halogen atom.

Another aspect of the invention provides immunogenic conjugates comprising the novel compounds and a protein, and monoclonal antibodies specific for the novel compounds of the invention, their protein conjugates, reductive byproducts, and adducts formed between mammalian tissue proteins and the compounds of the invention. The protein conjugates, reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention may be referred to generally as compositions. Methods for preparing the monoclonal antibodies are also provided. As will be appreciated, the monoclonal antibodies of the invention can be either to the novel compounds *per se* or to the compounds bound to a protein.

In a further aspect of the invention, methods for assaying tissue hypoxia are provided. A tissue sample may be assayed using immunohistochemical techniques or imaging techniques. Imaging techniques may be used for non-invasive analysis.

Kits useful for diagnostic applications comprising the novel compounds or compositions are also within the ambit of the present invention. These kits include a drug formulation of a compound of the invention and immunochemical reagents. The compounds of the invention are very useful in detecting oxygen levels because of their dramatic specificity for hypoxic cells over normal, healthy, oxygenated tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the effect of carbonate ion on the kineticsj of EF1 synthesis from the mixture of Ebr1 and potassium-kryptofix fluoride in DMSO at 120°C.

Figure 2 represents the HPLC analysis of the product of EF1 synthesis in the presence of radioactive ^{18}F with simultaneous detection of absorbency at 325 nm (upper curve) and radioactivity (lower curve); peak at 11-12 min. represents EF1.

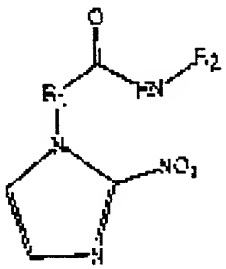
Figure 3 depicts the effect of relative flouride concentration on the EF1 yield.

Figure 4 illustrates a PET image of a tumor-bearing rat treated with 18-F-labeled EF1 (2-(2-nitro-1H-imidazol-1-yl)-N-3-monofluoropropyl) acetamide, 150 minutes post injection.

Figure 5 depicts a typical tissue section from the tumor of Figure 4 stained with anti-EF3 antibodies and imaged by fluorescence microscopy as previously described. *See* Evans *et. al*, *Brit J. Cancer*, **1995**, *72*, 875-882.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides a novel class of 2-nitroimidazole derivatives that are predictable oxygen indicators using both immunohistochemical assays and imaging techniques, said compounds having the structure:



wherein R_1 is CH_2; and R_2 has the formula $CH_2CX_2CHX_2$, wherein X is halogen or hydrogen and at least 1 carbon atom of said R_2 group is substituted with at least one halogen atom.

Preferred compounds of the invention may be viewed as pairs of, for example, brominated precursor and final product. For example, in certain preferred embodiments R_2 is $CH_2CH_2CH_2Br$ or $CH_2CH_2CH_2F$. In other preferred embodiments, R_2 is CH_2CH_2CHFBr or $CH_2CH_2CHF_2$. In yet other preferred embodiments, R_2 is $CH_2CF_2CH_2Br$ or $CH_2CF_2CH_2F$. And, in still other preferred embodiments, R_2 is CH_2CF_2CHFBr or in $CH_2CF_2CHF_2$. Also, in certain preferred embodiments where non-invasive imaging is used, one of the halogen atoms may be radioactive fluorine (^{18}F), having arisen from a precursor with bromine.

It is also believed to be possible to add fluorine gas across a double bond between the second and terminal carbon, leading to the possibility of only a single fluorine at the second carbon. Thus, in still other preferred embodiments, R_2 is CH_2CHFCH_2F, $CH_2CHFCHF_2$.

Because of the inherent difficulties in fluorine chemistry and exchange reactions it may be that other precursor molecules and final products of the general type specified may be most efficacious. It is believed that all molecules of this sort will have similar oxygen detection characteristics, the optimal compound is likely to be that which has the greatest efficiency of synthesis in radioactive form. Such compounds are contemplated to be within the scope of the claimed invention.

This invention is further directed to drug-protein conjugates (immunogenic conjugates) formed between a compound of the invention and a suitable carrier protein, these compositions may be referred to as antigens in this application. Proteins suitable for practicing this aspect of the invention include, without limitation, albumin, lysozyme (LYZ), or Bowman Birk inhibitor (BBI). In certain preferred embodiments, the immunogenic conjugates may have an R_2 as described above together with BBI. For example, R_2 may be $CH_2CF_2CH_2F$; $CH_2CH_2CH_2F$; or $CH_2CF_2CHF_2$.

The invention also presents methods for preparing a monoclonal antibody, which comprises introducing into a mammal a protein conjugate of the invention; fusing immune cells of the mammal with mammalian myeloma cells forming a hybridoma that produces antibodies specific for the compound bound to the protein. Monoclonal antibodies are also within the ambit of this invention.

In certain preferred embodiments, the protein is albumin, lysozyme, or Bowman Birk inhibitor and R_2 may be $CH_2CF_2CH_2F$; $CH_2CH_2CH_2F$; or $CH_2CF_2CHF_2$.

In one preferred embodiment of the invention, monoclonal antibodies will be specific for compounds and compositions of the invention where the halogen atom(s) are fluorine.

Methods for detecting tissue hypoxia are also presented. Imaging methods comprise using the novel compounds of the invention with or without immunohistochemical assays, preferably without the use of monoclonal antibodies to detect hypoxic cells.

In a noninvasive assay, the mammal is administered a compound of the invention, dissolved or dispersed in a suitable pharmaceutical carrier or diluent such as non-pyrogenic physiological saline. Any such diluents known to those skilled in the art may be used without departing from the spirit of the invention. The compound is allowed to partially clear from the mammal and to be taken up preferentially through the bioreductive metabolism of hypoxic cells, and then a portion of the mammal containing the tissue of interest is analyzed non-invasively such as through magnetic resonance imaging (MRI) or positron emission tomography (PET). A proportion of the compound will remain in the body, bound or associated with hypoxic cells. Tissue hypoxia is assayed using detectors of the marker atoms. Tissue hypoxia is assayed using detectors of the marker atoms. In the case of MRI, conventional non-radioactive (^{19}F) isotopes of fluorine are used. In the case of PET, a compound of the invention must first be formulated with the positron emitting isotope ^{18}F. Because of the short half-life of radioactive fluorine (110 min) a compromise must be reached between having the maximum clearance (providing the best signal: noise ratio), and having enough signal to provide adequate image resolution.

Imaging techniques suitable for practicing the invention include, but are not limited to, single photon emission computed tomography (SPECT), PET, and nuclear magnetic resonance imaging, usually called MRI. Generally, imaging techniques involve administering a compound with marker atoms that can be detected externally to the mammal.

Particularly preferred imaging methods for practicing the claimed invention include, PET, SPECT, or MRI. When the detection technique is PET, it is preferred that R_2 is $CH_2CH_2CH_2{}^{18}F$. When the detection technique is MRI, it is preferred that R_2 is $CH_2CH_2CH_2{}^{19}F$. In certain preferred methods, the label is a positron or gamma emitting isotope.

In another embodiment of the invention, the assay methods use immunochemistry. Generally, immunohistochemistry involves staining cryosectioned tissue samples. These methods generally comprise administering to a mammal, as above, a compound of the invention; obtaining a tissue sample; and detecting the presence of adducts formed between cells of the sample and a compound of the invention by contacting the tissue sample with the invention's monoclonal antibodies associated with a detection system. The

mAb will be specific for the adduct; that is, the mAb will be specific for the adduct formed between tissue proteins and the compound previously administered. In other words, the compound selectively binds to the tissue proteins of hypoxic cells to form an adduct. A sample of tumor tissue is obtained and the degree of tissue hypoxia is determined by quantifying the level of antibody interaction with the cells such as by using enzyme linked immunosorbant assay (ELISA), microdialysis, immunohistochemical staining, or other immunological protocols. The degree of binding of the antibodies to the side chain of the adduct provides a measurement of the degree of hypoxia in the tumor tissue. In a preferred embodiment of the invention, the monoclonal antibodies of the invention can be used with cells or tissue sections fixed in paraformaldehyde.

Methods of obtaining tissue samples for analysis, include any surgical and nonsurgical technique known in the art. Surgical methods include, but are not limited to biopsy such as fine needle aspirate, core biopsy, dilation and curettage.

Immunohistological techniques suitable for practicing the invention include, without limitation, immunoblotting or Western blotting, ELISA, sandwich assays, fluorescence, biotin or enzymatic labeling with or without secondary antibodies.

In certain preferred embodiments, R_2 is $CH_2CH_2CH_2Br$ or $CH_2CH_2CH_2F$. In other preferred embodiments, R_2 is CH_2CH_2CHFBr or $CH_2CH_2CHF_2$. In yet other preferred embodiments, R_2 is $CH_2CF_2CH_2Br$ or $CH_2CF_2CH_2F$. And, in still other preferred embodiments, R_2 is CH_2CF_2CHFBr or $CH_2CF_2CHF_2$. In still other preferred embodiments, R_2 is CH_2CHFCH_2F, $CH_2CHFCHF_2$. In certain preferred embodiments, the isotope is ^{18}F.

For purposes of the current invention, mammals include, but are not limited to the Order Rodentia, such as mice; Order Logomorpha, such as rabbits; more particularly the Order Carnivora, including Felines (cats) and Canines(dogs); even more particularly the Order Artiodactyla, Bovines (cows) and Suines (pigs); and the Order Perissodactyla, including Equines (horses); and most particularly the Order Primates, Ceboids and Simoids (monkeys) and Anthropoids (humans and apes). The preferred mammals are humans.

The invention is further directed to pharmaceutical formulations of the novel drug compounds. In accordance with preferred embodiments, a compound of the invention is dissolved or dispersed in a pharmaceutically acceptable diluent. Preferred diluents are

non-pyrogenic physiological saline.

The invention is also directed to formulations of immunogenic conjugates comprising the novel drug compounds of the invention bound to a protein carrier and dissolved or dispersed in a diluent.

Diagnostic kits are also within the scope of this invention. Such kits may include monoclonal antibodies that can rapidly detect tissue hypoxia; and include a compound of the invention, individual or mixed monoclonal antibodies against adducts formed between a compound of the invention and tissue proteins; and detection moieties. Preferably, standards of manufactured protein adducts to be used as calibration sources for the assays are also included.

Due to the unusual chemical properties of the novel claimed multiply halogenated alkyl chains, new chemical methods were used to synthesize the claimed compounds because previous work done to produce molecules suitable for PET imaging have not involved structures of this type. In particular, the degree of halogen saturation on the terminal carbon was modified to allow fluorine for bromine substitution while minimizing bromine elimination and /or molecular destruction under conditions suitable for such substitution (hot DMSO with fluoride carrier). The modifications allow the production of EF5 analogs with sidechains ending in $-CH_2CH_2F$, $-CH_2CHF_2$, $-CHFCH_2F$, $-CHFCHF_2$, $-CF_2CH_2F$ and $-CF_2CHF_2$. In each case, the brominated precursor molecule will have one of the terminal fluorines substituted by bromine.

Generally, the compounds of the invention can be synthesized using various reaction conditions depending on the starting material and ultimate requirements. In general there are up to 4 steps of the synthesis. First, the starting material for all compounds can be 2-nitroimidazol-1[H]-yl)-acetic acid. The terminal part of the side chain, containing the R_2 group as specified above, is a derivative of propylamine, wherein the C_2 and C_3 position are modified to contain one or more bromines and/or fluorines, in the next step. In the third step, the substituted propylamine is conjugated to the 2-nitroimidazol-1[H]-yl)-acetic acid in a mixed anhydride reaction. A final step may include the radioactive fluorine for bromine exchange reaction to make an agent suitable for PET imaging. Making of PET isotope-containing derivatives requires rapid addition of the ^{18}F moiety followed by

immediate purification and use because of the short half-life of ^{18}F, 109.7 minutes.

Generally, the third step of the synthesis for compounds of the invention is performed under the following reaction conditions. The reaction may be performed in anhydrous aprotic solvent with low boiling point (tetrahydrofuran or acetonitrile) under argon in the presence of tertiary amine (N-methylmorpholine or triethylamine) by addition of *iso*-butylchloroformate. The acid derivative then undergoes nucleophilic substitution with a halogenated alkylamine at the acid's carbonyl group to yield a halogenated nitroimidazole acetamide. Other synthetic methods will be apparent to those skilled in the art and may be used without departing from the spirit of the invention.

The claimed novel sidechains of the invention may generally be fluorine derivatives of propylamine. It is contemplated that these novel sidechains may be introduced into other compositions and compounds other than 2-nitroimidazole acetamide, including, without limitation, antibodies, receptors, protein conjugates, and the like. To make such compounds or compositions PET agents, ^{18}F is introduced into analogous compounds with bromine instead of fluorine. Generally, such a method would include conjugating a propylamine-based side chain with a carboxyl group of the compound or composition of interest (R_3COOH), forming R_3CONHR_2, where R_2 may be $CH_2CX_2CHX_2$. The next step is the introducing of ^{18}F by the exchange with bromine, as described, for example, in example 10. Any such compounds or compositions containing the novel sidechains of the invention are contemplated to be within the scope of the invention, as are the methods for making the same.

The reaction may yield a reaction slurry from which the product must be recovered. Methods of recovering the sample include any filtration or separation techniques known in the art. Such methods include, but are not limited to, vacuum filtration, separatory extraction, or distillation. A preferred method is filtration using air or liquid, but other methods will be apparent to those skilled in the art.

The filtration solid may further require washing with organic solvents to separate out impurities or other reaction intermediates or byproducts. Organic solvents include, but are not limited to, ether, methanol, ethanol, ethyl acetate, or hexanes. Ether is a preferred solvent, but other types of solvents will be apparent to those skilled in the art. Any organic solvent should be evaporated using methods known in the art. Evaporation methods

may be accomplished at room temperature, by vacuum, aspiration, or by using latent heat. The evaporation methods are not limited to these techniques and other techniques will be apparent to those skilled in the art.

The reaction product is then purified using purification techniques known in the art. These techniques include, but are not limited to, column chromatography, flash chromatography, recrystillization, or gel chromatography. When using chromatographic purification methods, gradient elution is preferred. Combinations of organic solvents include, but are not limited to, methanol, acetonitrile, hexanes, carbon tetrachloride, and ethyl acetate. Other purification methods will be apparent to those skilled in the art.

This invention is further directed to drug-protein conjugates formed between a compound of the invention and a suitable carrier protein, these compositions are referred to as antigens throughout this application. Antigens prepared using technology known in the art did not produce active mAbs, so previous procedures were substantially modified.

The prior art relates that antigen-forming reactions may be carried out between pH 4 to 7. It has now been found that these conditions fail to produce a sufficient number of drug-protein conjugates. It is greatly preferred to carry out the antigen-forming reactions at neutral or higher pH, preferably near neutrality. Under these conditions the drug-protein conjugation is much more efficient.

The conjugation process is also much more efficient when the carrier protein contains cysteine sulfhydryl groups (PSH). Unfortunately, the cysteine residues of most proteins are a) few in number (e.g., hemocyanin);b) are not accessible (e.g., alcohol dehydrogenase); or c) are oxidized as cystine dimers which do not bind reduced nitroaromatics. Although cystine dimers of several proteins can be very efficiently reduced via a radiochemical chain reaction, Koch & Raleigh, *Arch. Biochem. Biophys.*, **1991**,287, 75, the resulting modified protein is often insoluble possibly because of the formation of disulfide bridges between molecules. It was not possible to reduce the protein cystines by addition of excess quantities of agents such as dithiothreitol or mercaptoethanol, which can simultaneously reduce and stabilize cystine-containing proteins, because then adducts would preferentially form with the excess low-molecular weight thiol. Thus it was convenient to identify a protein with high cystine content, and having relative freedom from precipitation on

radiochemical reduction. Bowman Birk Inhibitor, a trypsin/chymotrypsin inhibitor from soybeans, (Bowman Birk Inhibitor (BBI) - 7 cystine bridges, molecular mass 7800) was found to have near optimal characteristics from this point of view, and reduction of up to an average of 8 cysteine residues was possible. The EF5-BBI conjugates were then made in a second radiochemical reduction step. Oxygen is excluded from the solutions using techniques previously described in Koch & Raleigh, Arch. *Biochem. Biophys.*, *supra*. Glass containers with specially constructed ceramic-enclosed spin bars to eliminate oxygen released from Teflon, Franko, *et al.*,"*Recent Results in Cancer Res. 95" in Culture of Cellular Spheroids* 62 (Verlag 1984), were placed into leak proof aluminum chambers, and the oxygen-containing air was replaced by nitrogen using a number of gas exchanges.

The monoclonal antibodies of the invention may be synthesized using the drug-protein conjugate of the invention. These conjugates are prepared according to the aforementioned procedure and are used to elicit antibody formation. When a drug-protein conjugate of the invention is bound to a protein carrier *in vitro* and administered to a mammal, monoclonal antibodies specific for compounds of the invention, their protein conjugates, reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention can be raised. The preparation of monoclonal antibodies is known in the art. Particularly, Kohler and Milstein's method, Kohler, *et al.*, *Nature*, **1975**, *256*, 495, with modifications as described in Knauf, *et al.*,*Cancer Immunol. Immunotherapy*, **1986**, *21*, 217-225.

Generally, drug-protein conjugate compositions would be used to immunize mice using conventional techniques. *See generally Knauf, et al., supra.* A host is injected with a drug-protein conjugate of the invention, serving as antigen to elicit an immune response. After an appropriate incubation period, blood would be drained from the host and analyzed. If the host's serum shows strong activity against the antigen, the animal would be sacrificed and its spleen cells used to make hybridoma clones. Kohler, *et al.*, *supra*. Such hybridomas are capable of producing monoclonal antibodies specific for the drug of the particular drug-protein conjugate administered to the mammal. Kohler, *et al.*, *supra*. In a preferred embodiment of the invention, the hybridoma clone will be conditioned to grow in serum-free medium. This ability to grow in serum-free medium permits facile purification of

the antibodies and the easy addition of detection moieties as a fluorophore, biotin, or an enzyme.

The drug compounds of the invention are very useful in detecting oxygen levels because of their dramatic specificity for hypoxic cells over normal healthy oxygenated tissue. For example, when hypoxic cells and aerobic cells are incubated in the presence of the new novel compounds, the monoclonal antibodies of the invention selectively bind to hypoxic cells. This preferential binding provides the basis for assaying tissues in mammals using immunohistological techniques.

The compounds of the invention possess unique properties that make them safer and more predictable oxygen indicators than previous compounds. The structure of the parent 2-nitroimidazole, etanidazole, N-(2-hydroxyethyl)-2(2-nitro-1H-imidazol-1-yl) acetamide, has been shown to be less susceptible to non-oxygen-dependent variations in adduct formation than is misonidazole. Also, the increased solubility of the compounds of the invention over misonidazole derivatives currently in use permits administering a higher drug concentration resulting in enhanced detection sensitivity without the toxicity observed with current methods.

It is believed that because the side chains of the claimed compounds of are highly non-physiological they will exhibit good antigenic characteristics. Monoclonal antibodies of this invention would be specific for the novel nitroaromatic compounds of the invention, their protein conjugates, their reductive byproducts, and adducts formed between mammalian hypoxic cells and the compounds of the invention. This specificity would make these antibodies superior detectors than the polyclonal antibodies currently used in the art. As indicated above, a consistent source of identical antibodies is required for clinical assays. The novel compounds of the invention provide the basis for a sensitive, versatile, and more accurate method for detecting tissue hypoxia.

Preferred aspects of the invention are discussed in the following examples. While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the invention is not so limited.

EXAMPLE 1

Synthesis of 2,2,3,3,3-pentafluoropropylamine (for making EF5)

Obtained commercially (PCR, Inc., P.O. Box 1466, Gainesville, FL 32602)

EXAMPLE 2

Synthesis of 3-bromo-2,2,3,3-tetrafluoropropylamine

3-bromo-2,2,3,3-tetrafluoropropylamine was prepared through the intermediate of 4-bromo-4,4,3,3-tetrafluorobutanoic acid (from literature: Wei Yuan, H.,Long, L., and Yuan-Fa, Z, *Chinese J. Chemistry* **1990,** *3*, 281). The reactions can be described by the following scheme:

$$BrCF_2CF_2Br + CH_2{=}CHOEt \xrightarrow{Na_2S_2O_4/NaHCO_3 \text{ in } CH_3CN/H_2O} BrCF_2CF_2CH_2CHBrOEt$$

$$\xrightarrow[CrO_3/H_2SO_4 \text{ in } CH_3COCH_3,]{} BrCF_2CF_2CH_2COOH \xrightarrow{NaN_3/H_2SO_4;\ HCl} BrCF_2CF_2NH_3Cl$$

$BrCF_2CF_2COOH$ (1.2 g, 5 mmol) was dissolved in 3 ml of H_2SO_4. Sodium azide (0.8 g, 12 mmol) was added in portion to the mixture at 80°. After addition was completed the reaction was continued for 20 hr. The mixture was then cooled to 0°. The solution was diluted with dichloromethane and then sodium carbonate (4 g in 20 ml of water). The organic layer was separated and the water layer was extracted with CH_2Cl_2 (20 ml x 2). The combined dichloromethane was dried over magnesium sulfate overnight and gaseous HCl bubbled into the solution. 0.79 g of white solid was collected by filtration and vacuum dried. ^1H NMRδ 3.82 (t, J = 16 Hz, 2H). ^{19}F NMR δ - 66..8 (t, J = 16 Hz, 2H),-113.74 (m, 2F). Chemical analysis: Calculated for $C_3H_5BrClF_4BN$ C: 14.6, H: 2.03, N:5.68. Found C: 14.57, H: 1.96, N: 5.56.

EXAMPLE 3

Synthesis of 3,3,3-trifluoropropylamine (for making EF3)

3,3,3-trifluoropropylamine hydrofluoride can be prepared in one step by treatment of 3-aminopropionic acid with excess SF_4 in anhydrous HF at 180°C. The product

can be converted to the hydrochloride by subsequent treatment with 40% KOH followed by an excess of HCl.

EXAMPLE 4

Synthesis of 3-bromo-3,3-difluoropropylamine

3-bromo-3,3-difluoropropylamine was prepared through the intermediate of 3-bromo-3, 3-difluoropropylazide according to the following, reaction schemes:

$$CF_2Br_2 + CH_2{=}CH_2 \rightarrow BrCF_2CH_2CH_2Br$$

$$BrCF_2CH_2CH_2Br + NaN_3 \rightarrow BrCF_2CH_2CH_2N_3$$

$$BrCF_2CH_2CH_2N_3 + PPh_3 + H_2O \rightarrow BrCF_2CH_2CH_2NH_2$$

3-bromo-3,3-difluoropropylazide was made by adding sodium azide (5 g, 77 mmol) and 1,3-dibromo-1,1-difluoropropane (12g, 50 mmol) in 50 ml DMSO. The mixture was stirred for 6 h at room temperature. After purification, 6.3 g of product was obtained. ^{1}H NMR δ 2.59 (m, 2H), 3.51 (t, J = 7 Hz, 2H). ^{19}F NMR δ -49.07 (t, J = 12 Hz, 2F). HRMS for $C_3H_4BrF_2N3$ Calc. 198.9557,200.9537. Found 198.9555, 200.9523.

Then, 3-bromo-3,3-difluoropropylamine was made by combining triphenylphosphine (2.62 g, 10 mmol), THF (10 ml) and water (1 ml) in a 50ml round bottom flask. 3-bromo-3,3-difluoropropylazide (1g, 5 mmol) was added dropwise to The mixture at 0°. After addition, the mixture was allowed to stir for an additional 6 hours. The product in THF was obtained by vacuum transfer. Most of the THF was removed by rotary evaporation. The residue was diluted by diethylether, and the ether layer dried over magnesium sulfate overnight. To prepare the hydrochloride, HCl was bubbled into the solution. The white solid (0.21 g) was obtained after filtration and vacuum dried.

^{1}H NMR δ 2.80 (m, 2H), 3.23 (t, J = 7 Hz, 2H). ^{19} F NMR δ -43.13 (t, J = 12 Hz, 2F). Anal. Calcd. for $C_3H_7BrC1F_2N$ C:17.1 H: 3.33 N: 6.65. Found C:17.12 H: 3.23 N: 6.48.

EXAMPLE 5

Synthesis of 3,3-difluoropropylamine

Synthesis of this compound uses 3-bromo-3,3difluoropopylazide (described above) as starting material. 5 g (2.5 mmol) of 3-bromo-3,3difluoropopylazide was added to benzene (10 ml)under nitrogen in combination with tributyltin hydride (2.91 g, 10 mmol).The mixture was refluxed for 8 h. The product went with benzene by vacuum transfer and following bubbling with HCl a white precipitate appeared. This was filtered and dried under vacuum to provide the final compound (0.51 g). ^{1}H NMR δ 2.20 (m, 2H), 3.13 (t, J = 7 Hz, 2H), 6.03 (t of t, J = 56 Hz, J = 4 Hz, 1H). ^{19}F NMR δ -115.52 (d of t, J = 56Hz, J = 18 Hz,2F). Anal. Calcd. for $C_3H_8BrClF_2N$ C:27.38 H: 6.08 N: 10.65. Found C: 27.45 H: 6.31 N: 10.42.

EXAMPLE 6

Synthesis of 3-fluoropropylamine

3-fluoropropylamine hydrochloride was prepared through the intermediate 3-fluoropropylazide according to the following reaction scheme:

$FCH_2CH_2CH_2Br + NaN_3 \rightarrow FCH_2CH_2CH_2N_3 + Na$

$FCH_2CH_2CH_2N_3 + PPh_3 \rightarrow FCH_2CH_2CH_2NH_2 \rightarrow FCH_2CH_2CH_2NH_3Cl$

Sodium azide (1g, 15 mmol) was stirred at room temperature with 15 mL of DMSO until most of sodium azide was dissolved. Then $FCH_2CH_2CH_2Br$ (1.41 g, 10 mmol) was added to the mixture and continued stirring for 6 hours. The crude product (0.85 g, 83%) was obtained by vacuum transfer. ^{1}H NMR δ 1.23 (m, 2H), 2.72 (t, J = 7 Hz, 2H), 3.93 (d of t, J = 47 Hz, J =6 Hz, 2H). ^{19}F NMR δ -222.80 (m, 1F)

Triphenylphosphine (2.62 g, 10 mmol) was dissolved in 8 mL of THF, then 3-fluoropropylazide (0.85 g, 8.3 mmol) was added dropwise to the solution at 0°C. After addition, the mixture was warmed to room temperature slowly and stirred for an additional 6 hours, then water (0.22g, 12 mmol) was added to the solution. The mixture was stirred at room temperature overnight. The product in THF was obtained by vacuum transfer and was acidified with dry hydrogen chloride. The white precipitate was filtered to provide 0.63 g (48%) of product. ^{1}H NMR d 1.95 (m, 2H), 3.04 (t, J = 7 Hz, 2H), 4.50 (d of t, J = 47 Hz, J =5 Hz, 2H). ^{19}F NMR d

-219.70 (m, 1F).Analysis: calculated for C_3H_5ClFN C:31.72, H: 7.93, N 12.33; found C:31.56, H: 8.20, N 11.83

EXAMPLE 7

Synthesis of 3-bromo-2,2-difluoropropylamine

The reaction scheme is analogous to the synthesis of 3-bromo-2,2,3,3-tetrafluoropropylamine (see Example 2). In this synthesis $BrCF_2CH_2Br$ is using as a starting material instead of $BrCF_2CF_2Br$, leading to synthesis of $BrCH_2CF_2CH_2COOH$ after oxidation of addition product by chromium (VI) oxide and $BrCH_2CF_2CH_2NH_3Cl$ after sodium azide treatment.

EXAMPLE 8

Synthesis of 3-bromopropylamine (for making EBr1)

Obtained commercially (Aldrich).

EXAMPLE 9

Optimization of the synthesis of EF1 from EBr1.

EF1 was prepared from EBr1 by the direct exchange of bromine with potassium-kryptofix [2,2,2] fluoride in DMSO. In typical preparation 100 μL of water, containing 7 μmol of potassium-kryptofix [2,2,2] fluoride and 1.5 μmol of potassium-kryptofix [2,2,2] carbonate were dried by azeotropic distillation with acetonitrile (3 ¥ 2 mL) at 120°C under stream of argon. Solution of 2.9 mg EBr1 (10 μM) in 1 mL of DMSO was added and the mixture was heated at 120°C for 40 min under nitrogen. The probes of solution were diluted 1:100 into 0.1 M ammonia-acetate buffer and analyzed by HPLC on C-18 column with elution by the same buffer with 10% methanol and detection of absorbency at 325 nm (for 2-nitroimidazole e=7,500). Comparison of HPLC data with standard solution shows the yield of EF1 approximately 2%, which may be considered acceptable for preparation of [^{18}F]-EF1.

To optimize the reaction conditions, the reaction conditions were varied. Addition of 10-fold excess of fluoride to any 2-nitroimidazole derivative at room temperature

caused a rapid change of yellowish color of solution to dark-blue and next brown. Absorption spectrum of product has no band at 325 nm, suggestion the decomposition of 2-nitroimidazole ring. Accordingly, an excess of fluoride can not be used for the reaction.

Presence of traces of water drastically reduced the yield of EF1 and causes a production of subsequent hydroxyl derivative. In order to prevent this effect, the anhydrous DMSO was preheated before the reaction at 120°C with bubbling of argon during 2 hours.

Preparation of [^{18}F] (see below) implies the presence of residual carbonate in solution. The effect of carbonate on the reaction kinetics was determined. The results (Fig 4) show, that optimal ratio of fluoride to carbonate is 4:1, which is consistent with data. Hamacher, *et al, J. Nuc. Med.*, **1986**, *27*, 238. Efficient stereospecific synthesis of no-carrier-added 2-[^{18}F]-fluoro-2-deoxy-D-glucose using aminopolyether supported nucleophilic substitution.

Different aprotic solvents were tested. The yield of EF1 was negligible in hexamethylphosphamide, 0.2% in dimethylformamide and 0.8% in dimethylimidazolinone. Subsequently, DMSO (2%) is the optimal solvent for the reaction, probably due to the most efficient ionization of F^- in solution.

EXAMPLE 10

Preparation of [^{18}F]-EF1

[^{18}F]-hydrofluoric acid was prepared by the $^{18}O(p,n)^{18}F$ reaction using ^{18}O-enriched water as the target material. The [^{18}F]-hydrofluoric acid (200 mCi) was mixed with 100 μL of water, containing 7 μmol of potassium-Kryptofix [2,2,2] fluoride and 1.5 μmol of potassium-Kryptofix [2,2,2] carbonate. The solution was dried by azeotropic distillation with acetonitrile (3 ¥ 2 mL) at 120°C, and solution of 2.9 mg EBr1 (10 μM) in 0.5 mL of DMSO was added. The solution was heated at 120°C for 40 min under nitrogen. The reaction vessel was cooled and 3 mL of water was added. In order to remove unreacted fluoride, the water solution was passed through the column, packed with Dowex 1X4-50 chloride. The yield of radioactive product was 1.5 mCi.

The probe of solution was analyzed by HPLC with simultaneous detection of 325 nm absorbency and radioactivity. As seen in Fig. 2, most of radioactivity is eluted as a

single peak, correspondent to 325 nm absorbency peak of EF1. Subsequently, the solution contains ^{18}F mostly in the form of [^{18}F]-EF1.

The described above procedure involves the addition of carrier ^{19}F to the reaction mixture. It seems to be more logical to use only radioactive fluoride to achieve higher degree of conversion of ^{18}F into [^{18}F]-EF1. However, attempts to use only ^{18}F without carrier resulted in the very low (if any) production of [^{18}F]-EF1. To explain this effect, the reaction was performed at fixed EBr1 concentration, decreasing the F-/EBr1 ratio. As it is shown in Fig 3, it also caused the decrease of the relative yield of EF1, as compared with fluoride. Subsequently, the decrease of fluoride concentration does not favor the conversion of fluoride into EF1, probably due to overwhelming by other reactions. Another explanation on the necessity of the carrier is very low concentration of ^{18}F in solution. High specific activity ($1.71 \cdot 10^{9}$ Ci/mmol) suggests the $6 \cdot 10^{-13}$ M concentration of fluoride in the reaction solution. At this low concentration the traces of water and other impurities may significantly affect the reaction, causing decrease of the [^{18}F]-EF1 yield.

EXAMPLE 11

PET Analysis of a Tumor-bearing Rat Treated with [^{18}F]-EF1

Figure 4 illustrates a PET image of a tumor-bearing rat treated with 18-F-labeled EF1 (2-(2-nitro-1H-imidazol-1-yl)-N-3-monofluoropropyl) acetamide, 150 minutes post injection.

Q7 cells were obtained from the American Type Culture Collection (ATCC). They were maintained in exponential growth by transfers at 3.5 day intervals with standard culture conditions. Growth medium was Eagle's MEM supplemented with 15% fetal calf serum and standard penicillin and streptomycin.

All animal studies conformed to the regulations of the University of Pennsylvania Institutional Animal Care and Use Committee. Male Buffalo rats (Harlan Sprague Dawley, Indianapolis, Indiana, USA) were used for all studies. Donor tumors were created by injecting 1 million Q7 cells subcutaneously into the thigh region. The average growth time to achieve a 1 cm diameter tumor was 21 days. Tumors of less than 2g were used in the experiments.

The tumor (Morris 7777 hepatoma)is clearly visible even though various organs also expected to bind the drug were nearby (liver, kidney, stomach, cecum, digestive track etc.). It is believed that this is the first PET image of a rodent tumor where substantial image modifications to eliminate gut clearance effects have not been necessary.

EXAMPLE 12

Analysis of Tissue Section from the Tumor of Figure 4 Stained with Anti-EF3 Antibodies and Imaged by Fluorescence Microscopy

Figure 5 depicts a typical tissue section from the tumor of Figure 4 stained with anti-EF3 antibodies and imaged by fluorescence microscopy as previously described. *See* Evans *et. al*, *Brit J. Cancer*, **1995**, *72*, 875-882. Since existing antibodies (to EF5 and EF3) have only a modest affinity towards EF1, the rat was simultaneously injected with EF3 to allow normal immunohistochemical staining of the tumor tissue. Q7 tumor sections were cut at 14μm thickness using a Microm HM 505N cryostat and collected onto poly-L-lysine coated microscope slides. The sections were fixed for one hour in ice cold Dulbecco's phosphate-buffered saline (1X PBS) containing freshly dissolved paraformaldehyde (4%m, pH 7.1-7.4, SIGMA P-6148). The rinsing, blocking and staining of tissue sections for EF3 binding was identical to that described previously.

EF3 binding was assessed by imaging the tissue sections at the appropriate wavelengths for EUL5-A8 (535nm excitation, 605nm emission). Slides were imaged using a Nikon fluorescence microscope fitted with either a standard camera back (for Ektachrome Elite 400 film) or digital CCD camera (Xillix Technologies, Vancouver). Preceding microscope use, the brightness of the fluorescent bulb was calibrated so that measurements of exposure times for individual tissue sections could be directly compared. EUL5-A8 dye with absorbency 1.25 at 549nm was loaded into a hemcytometer and the fluorescence recorded after focusing the microscope on the ruled grid of the hemocytometer. Image fields of 1.2 mm x 1.0 mm and 1.05 mm x 0.75 mm were obtained from the CCD and regular camera, respectively, for a 10x objective, and correspondingly larger fields for a 4x objective. Photography of EUL5-A8 conjugated antibody was made at noted vernier locations on the tissue section.

EXAMPLE 13

Analysis of the Distribution of Radioactive Drug in Various Organs and Tissues

To measure the distribution of radioactive drug in various organs and tissues, the solution of [^{18}F]-EF1 in saline buffer was injected I/V into 2 male Buffalo rats. Animals was sacrificed and the samples of tissues were collected and weighted. The radioactivity of samples was measured by γ-counter and corrected for weight and the time of decay.

Table 1 shows the actual distribution of radioactive counts from various organs and tissues after animal sacrifice and tissue collection. In particular, note that the density of radioactive counts closely parallels the findings from the image analysis. Results from 2 animals are shown. PET and immunohistochemical images from both animals were very similar (data not shown)

Table 1. Tissue distribution of [^{18}F]-EF1 in rats bearing tumors (%dose/gram).

Organ	3 hrs	3 hrs	4 hrs	4 hrs
Blood	0.31	0.12	-	-
Brain	0.13	0.11	-	-
Liver	0.25	0.21	0.41	0.19
Spleen	0.17	0.13	0.36	0.15
Kidney	0.54	0.29	0.67	0.31
Muscle	0.17	0.13	0.23	0.13
Tumor	0.34	0.28	0.64	0.44

EXAMPLE 14

Analysis of the Distribution of Radioactive Drug in Various Murine Organs and Tissues

The distribution of radioactive drug in various murine organs and tissues was measured similarly to the previous example. [^{18}F]-EF1 was injected into 4 mice, which were sacrificed after 5 and 90 minutes and the radioactivity of tissues was measured.

Table 2 shows the biodistribution of EF1 in various murine tissues at varying times after drug administration. The overall distribution of counts is quite similar to that found for radioactive EF5(^{14}C-labeled) except for brain. Mouse-brain tissue contained substantially lower densities of labeled EF1 at early times, compared with other organs. This finding is consistent with the expected hydrophilicity of EF1, compared with EF5.

Table 2. Distribution of [^{18}F]EF-1 in murine tissues.

Tissue	**5 minutes**	**5 minutes**	**90 minutes**	**90 minutes**
Blood	0.041	0.038	0.005	0.007
Brain	0.004	0.004	0.003	0.005
Muscle	0.038	0.031	0.005	0.009
Liver	0.064	0.050	0.016	0.020
Spleen	0.034	0.035	0.005	0.007
Kidney	0.072	0.044	0.011	0.015
Tibia	0.045	0.042	0.079	0.064
Cecum	0.022	0.023	0.021	0.045
Stomach	0.013	0.013	0.007	0.009
Intestine	0.037	0.039	0.014	0.012
Esophagus			0.012	
Urine	0.045		0.595	0.786
Tail	0.100	0.041	0.071	0.051
Lung	0.052	0.046	0.007	0.005
Heart	0.041	0.052	0.006	0.009

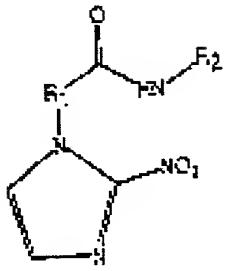
EXAMPLE 15

General Synthetic Method for Certain Compounds of the Invention

Brominated precursors to EF3 and EF5 wherein one of the terminal fluorines was substituted by bromine have now been made. Nucleophilic exchange reactions were attempted using the conditions described in Example 10, but problems arose because of the unusual chemical properties of multiply halogenated alkyl chains. The problems were diametrically opposed for the two precursors. For the EF3 precursor, named EF2Br (sidechain ending in-CH_2CF_2Br), rapid bromine elimination occurred because of the ease with which hydrogen can be co-eliminated from the adjacent carbon. For the EF5 precursor, named EF4Br (sidechain ending in CF_2CF_2Br) the bromine-carbon bond is highly stabilized and exchange conditions must be sufficiently harsh that the core 2-nitroimidazole structure is destroyed. The invention employs new chemical methods because previous work done to produce molecules suitable for PET imaging have not novel involved structures of the kind claimed. Essentially, the degree of halogen saturation on the terminal carbon has been modified to allow fluorine for bromine substitution while minimizing bromine elimination and /or molecular destruction under conditions suitable for such substitution (hot DMSO with fluoride carrier).

What is claimed is:

1. A compound having the formula:



wherein R_1 is CH_2; and R_2 has the formula $CH_2CX_2CHX_2$, wherein X is halogen or hydrogen and at least 1 carbon atom of said alkyl group is bound with at least one halogen atom.

2. The compound of Claim 1 wherein the halogen atom is fluorine.

3. The compound of Claim 1 wherein the halogen atom is bromine.

4. The compound of Claim 1 wherein R_2 is $CH_2CH_2CH_2Br$.

5. The compound of Claim 1 wherein R_2 is $CH_2CF_2CH_2Br$.

6. The compound of Claim 1 wherein R_2 is CH_2CF_2CHFBr.

7. The compound of Claim 1 wherein R_2 is $CH_2CF_2CHBr_2$.

8. The compound of Claim 1 wherein R_2 is $CH_2CF_2CH_2F$.

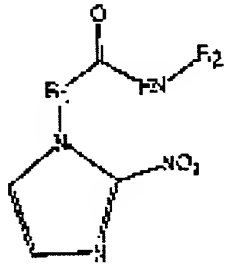
9. The compound of Claim 1 wherein R_2 is $CH_2CF_2CH_2F$.

10. The compound of Claim 1 wherein R_2 is $CH_2CF_2CHF_2$.

11. The compound of Claim 1 wherein R_2 is CH_2CHFCH_2F

12. The compound of Claim 1 wherein R_2 is $CH_2CHFCHF_2$

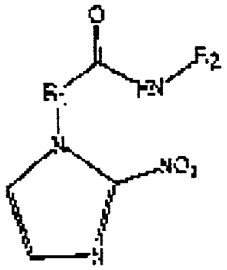
13. A compound bound to a protein, the compound having the formula:



wherein R_1 is CH_2; and R_2 has the formula $CH_2CX_2CHX_2$, wherein X is halogen or hydrogen and at least 1 carbon atom of said alkyl group is bound with at least one halogen atom.

14. A method for preparing a monoclonal antibody comprising:

introducing into a mammal a compound bound to a protein, the compound having the formula:

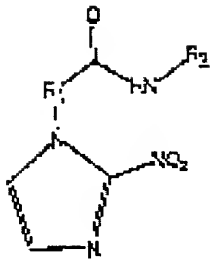


wherein R_1 is CH_2; and R_2 has the formula $CH_2CX_2CHX_2$, wherein X is halogen or hydrogen and at least 1 carbon atom of said alkyl group is bound with at least one halogen atom; and

fusing immune cells of the mammal with mammalian myeloma cells forming a hybridoma that produces antibodies specific for the compound bound to the protein.

15. The method of claim 14 wherein R_2 is $CH_2CH_2CH_2F$.

16. A monoclonal antibody specific for a compound, the compound's protein conjugate, the compound's reductive byproduct, or adduct formed between The compound and tissue protein, the compound having the formula:



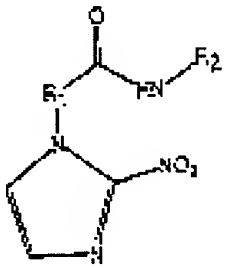
wherein R_1 is CH_2; and R_2 has the formula $CH_2CX_2CHX_2$, wherein X is halogen or hydrogen and at least 1 carbon atom of said alkyl group is bound with at least one halogen atom.

17. The monoclonal antibody of claim 16 wherein the halogen atom is fluorine.

18. The monoclonal antibody of claim 16 wherein R_2 is $CH_2CH_2CH_2F$.

19. A biological reagent kit comprising the monoclonal antibody of claim 16 bound to a detection moiety.

20. A method for detecting tissue hypoxia in a mammal comprising: introducing into the mammal a compound having the formula:



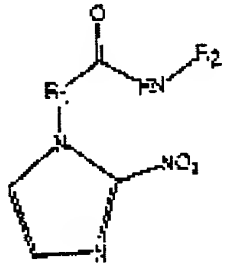
wherein R_1 is CH_2; and R_2 has the formula $CH_2CX_2CHX_2$, wherein X is halogen or hydrogen and at least 1 carbon atom of said alkyl group is bound with at least one halogen atom; and

imaging the portion of the mammal containing the tissue.

21. The method of claim 20 wherein the detection technique is PET.

22. The method of claim 20 wherein R_2 is $CH_2CH_2CH_2{}^{18}F$ and the detection technique is PET.

23. A kit for detecting tissue hypoxia comprising a compound having the formula:



wherein R_1 is CH_2; and R_2 has the formula $CH_2CX_2CHX_2$, wherein X is halogen or hydrogen and at least 1 carbon atom of said alkyl group is bound with at least one halogen atom; a protein; a monoclonal antibody specific for the compound the compound's protein conjugates, the compound's reductive by product, or adduct formed between the compound and tissue protein; standards comprising the compound bound to a protein; a monoclonal antibody bound to a detection moiety; and detection moieties.

24. The kit of Claim 23 wherein compound is bound to lysozyme, albumin, or Bowman Birk inhibitor.

25. The kit of Claim 23 wherein R_2 is $CH_2CH_2CH_2F$ and the protein is Bowman Birk Inhibitor.

26. The kit of Claim 23 wherein the detection moiety is a fluorophore, biotin, or an enzyme.

ABSTRACT

Novel nitroaromatic compounds and immunogenic conjugates comprising a novel nitroaromatic compound and a carrier protein are disclosed. The invention further presents monoclonal antibodies highly specific for the claimed nitroaromatic compounds, the compounds' protein conjugates, the compounds' reductive byproducts, and adducts formed between the compounds and mammalian hypoxic cell tissue proteins. The invention is further directed to methods for detecting tissue hypoxia using immunohistological techniques, non-invasive nuclear medicinal methods, or nuclear magnetic resonance. Diagnostic kits useful in practicing the methods of claimed invention are also provided.

Figure 1. Effect of $[KKrf]_2(CO_3)$ on kinetics of EF1 synthesis from 10 mM EBr1 + 10 mM [KKrf]F .

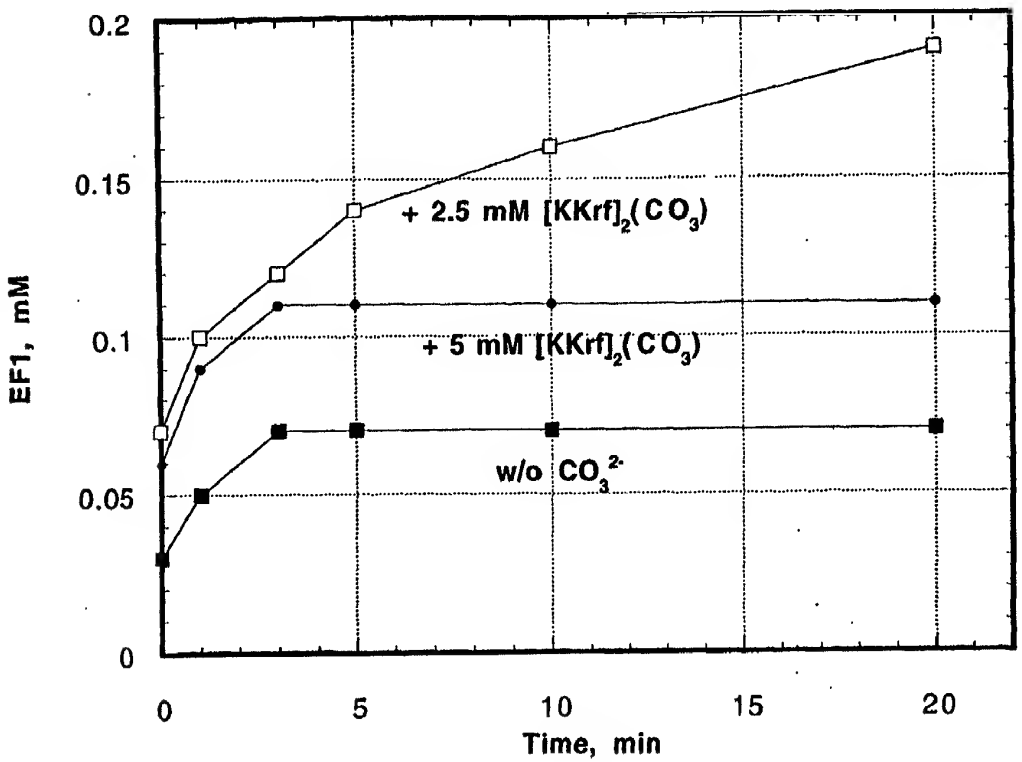


Figure 2. HPLC analysis of [^{18}F]EF-1 with simultaneous detection of 325 nm absorbency (upper curve) and radioactivity (lower curve).

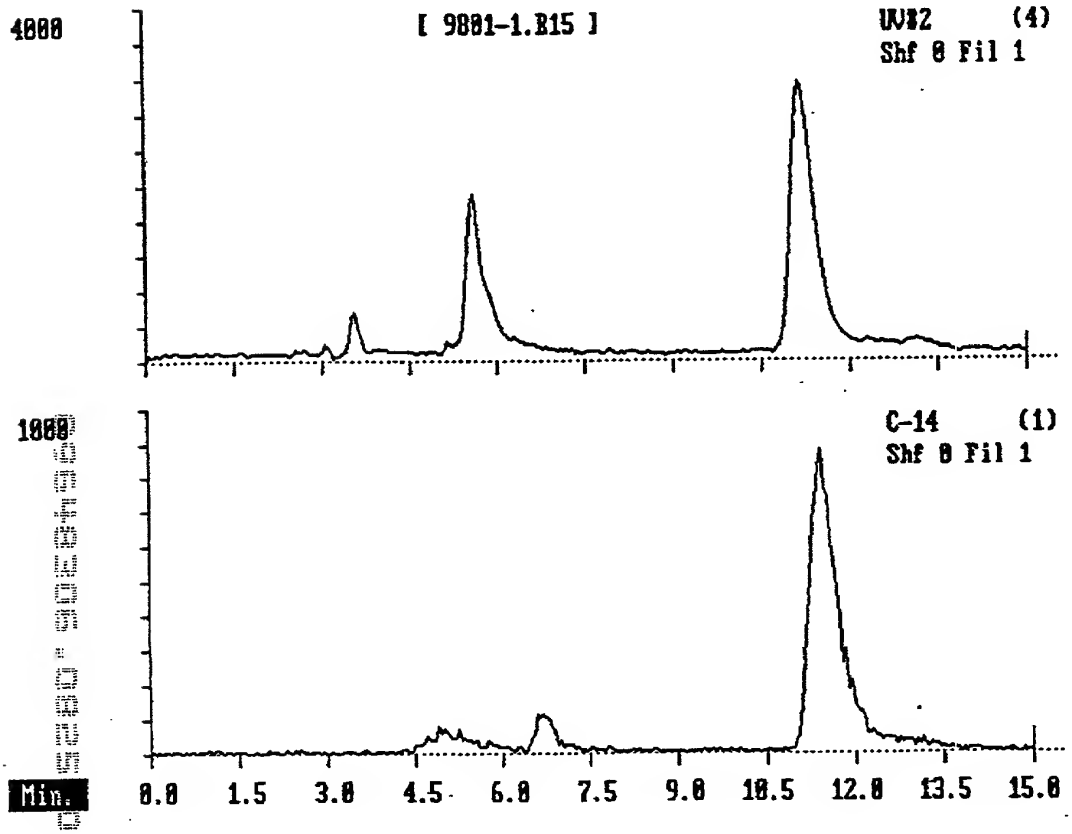
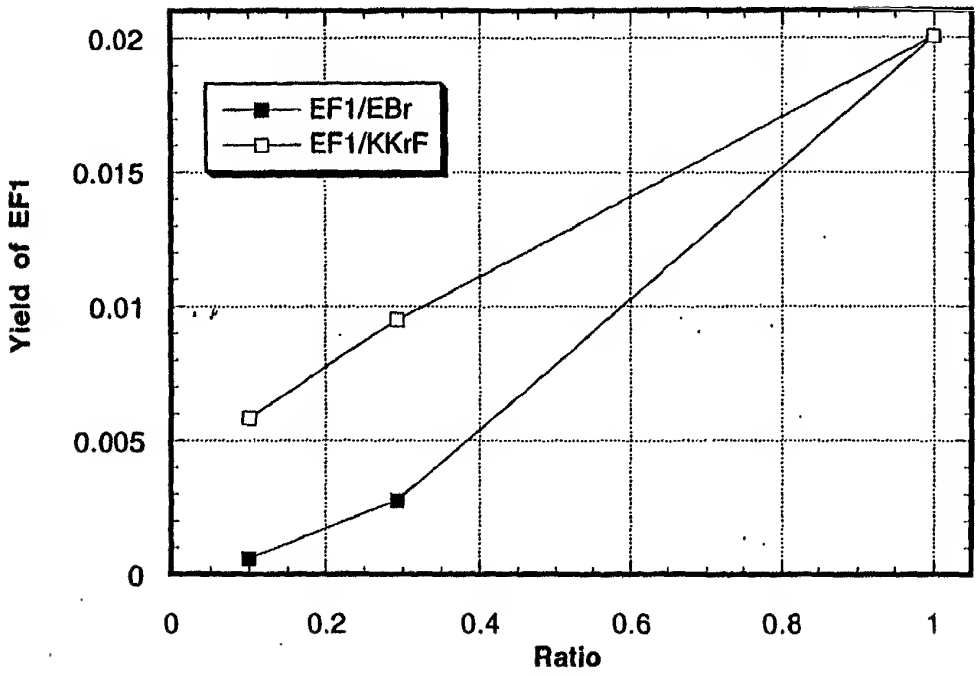


Figure 3. Effect of $[KKr]F_{0.7}(CO_3)_{0.15}$ to EBr1 ratio on the EF1 yield.



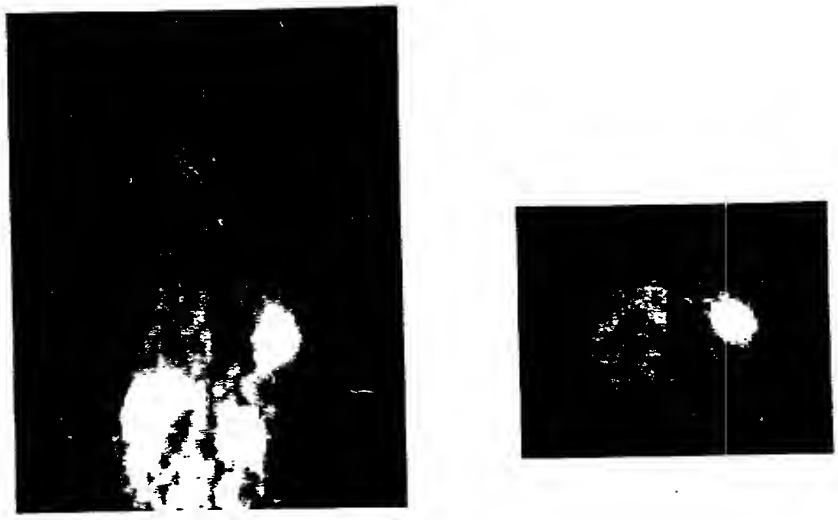


FIGURE 4

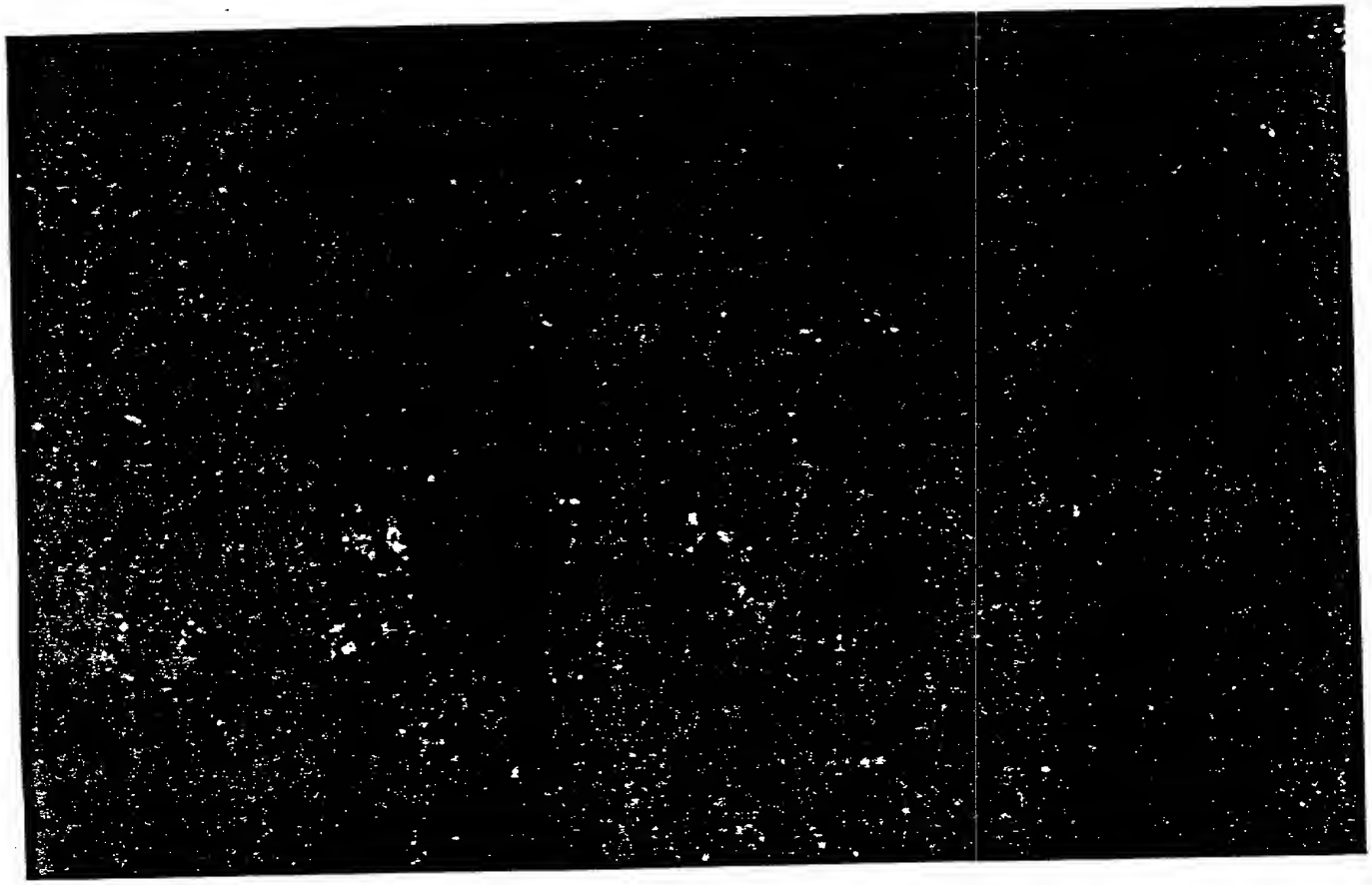


FIGURE 5

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:
Cameron J. Koch, Alexander V. Kachur, Sydney M. Evans, Chyng-Yann Shiue, Ian R. Baird, Kirsten A. Skov, William R. Dolbier, Jr., An-Rong Li, and Brian R. James

Group Art Unit: N/A

Examiner: N/A

For: DETECTION OF HYPOXIA

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and I believe that I am the original, first and joint inventor of the subject matter which is claimed and for which a

☒ Utility Patent ☐ Design Patent

is sought on the invention, whose title appears above, the specification of which:

☐ is attached hereto.

☒ was filed on July 28, 1998 as Serial No. 09/123,300 .

☐ said application having been amended on _____ .

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign application(s)** for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of

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Priority Claimed (If X'd)	Country	Serial Number	Date Filed
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Serial Number	Date Filed	Patented/Pending/Abandoned
08/598,752	February 8, 1996	U.S. Pat. 5,843,404

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Serial Number	Date Filed

I hereby appoint the following persons of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103 as attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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